



BEST AVAILABLE COPY

I hereby Certify that this Correspondence is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to:

Commissioner for Patents, P.O. Box 1450, Alexandria,
Virginia 22313-1450, on

Name

Shinichi Kanno

Signature

January 4, 2005

Date of Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No. : 09/858,036

Applicant(s) : Kanno, et al.

Filed : May 15, 2001

Title : Method For Inducing Angiogenesis By Electrical Stimulation Of
Muscles

Docket No. : 31090.0015

37 C.F.R. 1.131 Declaration

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The undersigned, Shinichi Kanno, declares:

1. That he is Assistant professor of Surgery at the University of Pittsburgh School of Medicine.
2. That he is the inventor on the above-referenced patent application.
3. That he is familiar with the Office Action mailed on October 4, 2004 in which claims 1-15 were rejected as anticipated by Donovan et al. (Patent Application

Rule 1.131 Declaration by Dr. S. Kanno
Page 2

Publication No. 2002/0010492), which claims priority to Provisional Application No. 60/187,280, filed March 6, 2000.

4. That the present invention relating to a method for inducing angiogenesis in a muscle tissue was made at least as early as May 25, 1999, as evidenced by the accompanying publication (Kanno, et al. *Circulation*. (May 25, 1999) 99:2682-2687) which discloses the method of the invention and its usefulness in 1) increasing VEGF mRNA expression in a muscle cell (i.e., see Figure 3, page 2684), and 2) inducing angiogenesis in a muscle tissue, wherein the angiogenesis comprises an increase in capillary density (i.e., see Figure 7, page 2685).

5. That all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued there from.

Respectfully submitted,

12/20/04

Date

Shinichi Kanno

Dr. Shinichi Kanno

Establishment of a Simple and Practical Procedure Applicable to Therapeutic Angiogenesis

Shinichi Kanno, MD; Nobuyuki Oda, MSc; Mayumi Abe, MD, PhD; Sachiko Saito, PhD;
Katsuyoshi Hori, PhD; Yasunobu Handa, MD, PhD;
Koichi Tabayashi, MD, PhD; Yasufumi Sato, MD, PhD

Background—Therapeutic angiogenesis is thought to be beneficial for serious ischemic diseases. This investigation was designed to establish a simple and practical procedure applicable to therapeutic angiogenesis.

Methods and Results—When cultured skeletal muscle cells were electrically stimulated at a voltage that did not cause their contraction, vascular endothelial growth factor (VEGF) mRNA was augmented at an optimal-frequency stimulation. This increase of VEGF mRNA was derived primarily from transcriptional activation. Electrical stimulation increased the secretion of VEGF protein into the medium. This conditioned medium then augmented the growth of endothelial cells. The effect of electrical stimulation was further confirmed in a rat model of hindlimb ischemia. The tibialis anterior muscle in the ischemic limb was electrically stimulated. The frequency of stimulation was 50 Hz and strength was 0.1 V, which was far below the threshold for muscle contraction. After a 5-day stimulation, there was a significant increase in blood flow within the muscle. Immunohistochemical analysis revealed that VEGF protein was synthesized and capillary density was significantly increased in the stimulated muscle. Rats tolerated this procedure very well, and there was no muscle contraction, muscle injury, or restriction in movement.

Conclusions—We propose this procedure as a simple and practical method of therapeutic angiogenesis. (*Circulation*. 1999;99:2682-2687.)

Key Words: electrical stimulation ■ growth substances ■ angiogenesis

Arterial occlusive diseases cause serious ischemic diseases in various organs, such as the heart, brain, and leg, and therapeutic angiogenesis is thought to be beneficial for such conditions.¹ Local administration of recombinant angiogenic growth factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), salvaged ischemic areas of myocardium and hindlimb in animal models.²⁻⁶ However, the clinical application requires large amounts of these recombinant proteins and is not feasible at this time. Instead of recombinant proteins, use of gene therapy, ie, *in vivo* transfection of angiogenic growth factor gene, has been attempted to treat these diseases. In particular, the clinical trial of intramuscular gene transfer of naked plasmid DNA encoding human VEGF₁₆₅ is progressing in the United States for the treatment of ischemic limbs.⁷

See p 2614

VEGF, a dimeric endothelial cell (EC)-specific growth factor, is thought to be a principal angiogenic factor that stimulates migration, proliferation, and expression of various genes in ECs.⁸⁻¹⁰ VEGF is synthesized by cells around

vasculature and affects ECs as a paracrine factor. The expression of VEGF is upregulated by hypoxia and various cytokines. Our present study revealed that low-voltage electrical stimulation of skeletal muscle induced de novo synthesis of VEGF protein, promoted local angiogenesis, and restored blood flow in the ischemic area. We propose this procedure as a simple and practical method of therapeutic angiogenesis.

Methods

Cell Culture and Electrical Stimulation In Vitro

Murine skeletal muscle cell line C2C12 cells were obtained from Riken Cell Bank; rat aortic smooth muscle cells from Hanno Research Center, Taiho Pharmaceutical Co Ltd; and human pulmonary artery smooth muscle cells from Kurabo. Cells were routinely cultured on plastic dishes in DMEM (Nissui Pharmaceutical Co, Ltd) containing 10% FCS (Summit Biotechnology). Bovine capillary ECs (BCECs) were obtained from Dr T. Tamaoki (Kyowa Hakko Kogyo Co, Ltd) and were grown in DMEM containing 10% FCS as described previously.¹¹ All primary cultures of passage <6 were used in following experiments.

Received November 20, 1998; revision received February 2, 1999; accepted February 12, 1999.

From the Department of Vascular Biology, Institute of Development, Aging, and Cancer, Tohoku University (S.K., N.O., M.A., S.S., K.H., Y.S.); the Department of Restorative Neuromuscular Surgery and Rehabilitation, Tohoku University Graduate School of Medicine (Y.H.); and the Department of Thoracic and Cardiovascular Surgery, Tohoku University School of Medicine (S.K., K.T.), Sendai, Japan.

Correspondence to Yasufumi Sato, MD, PhD, Department of Vascular Biology, Institute of Development, Aging, and Cancer, Tohoku University, 4-1 Seiryomachi, Aoba-ku, Sendai, 980-8575, Japan. E-mail y-sato@idac.tohoku.ac.jp

© 1999 American Heart Association, Inc.

Circulation is available at <http://www.circulationaha.org>

Electrical Stimulation of Cells In Vitro

Before the following experiments, cells were preincubated for 24 hours in DMEM containing 0.1% BSA. Confluent cultures were electrically stimulated at 1.0 V of stimulus strength and at indicated frequencies (Hz) for an indicated period, according to the method described by Brevet and Pinto.¹²

Northern Blot Analysis

Northern blot analysis was carried out as described previously.¹³ Briefly, total RNA was extracted by the Acid Guanidium-Phenol-Chloroform method and fractionated on a 1% agarose gel containing 2.2 mol/L formaldehyde. The blots were then prepared by transfer onto a nylon filter (Hybond N⁺, Amersham). The filter was hybridized with a ³²P-labeled probe in hybridization solution for 24 hours at 42°C. After the hybridization, the filter was washed in 2×SSC and 0.1% SDS at 60°C and then in 0.2×SSC and 0.1% SDS at 60°C. Autoradiography was carried out on an imaging plate, and autoradiograms were analyzed with an FLA 2000 image analyzer (Fuji). Human GAPDH cDNA templates were prepared as previously described.¹³ Flk-1 and VEGF cDNA templates were prepared by reverse-transcription polymerase chain reaction using the following primer pairs: Flk-1: sense, 5'-AGGGGAACGTGAAAGACAGG-CTA-3' and antisense, 5'-GATGCTCCAAGGTGAGGAAGT-3'; VEGF: sense, 5'-GTGCACTGGACCCTGGCTTT-3' and antisense, 5'-AAGCTGCCTCGCCTTGCAAC-3'.

Measurement of VEGF Protein in Conditioned Medium

C2C12 cells in DMEM containing 0.1% BSA were electrically stimulated for 24 hours at 1.0 V and 50 Hz, and the medium was collected. VEGF protein in the medium was measured by Quantikine-M ELISA kit (R&D Systems) according to the manufacturer's protocol.

Effect of Medium Conditioned by Electrical Stimulation on the Growth of BCECs

BCECs (5×10^4) were plated on plastic dishes in DMEM with 10% FCS and incubated for 6 hours to allow attachment to the dish. The culture medium was replaced with either 100% conditioned medium, fresh medium (DMEM containing 0.1% BSA), or fresh medium supplemented with 10 ng/mL of recombinant human VEGF. Cell numbers were counted after 48 hours of incubation.

Electrical Stimulation In Vivo

All animal studies were performed according to a protocol approved by the Animal Experiment Committee of our institution. Male Sprague-Dawley rats (300 to 350 g body weight) were used. The animals were anesthetized with light ether sedation and subcutaneous injection of pentobarbital sodium (50 mg/kg) (Nembutal, Abbott Laboratories). The operation for hindlimb ischemia was performed according to the method described by Takeshita et al.¹⁴ Briefly, the left femoral artery (FA) was completely excised from its proximal origin to the point distally at which it bifurcates into the saphenous and popliteal arteries. After 1 week was allowed for recovery from the operation, blood flows of the bilateral tibialis anterior (TA) muscles were measured by the hydrogen gas clearance technique originally described by Aukland et al¹⁵ and modified by Hori et al.¹⁶ Thereafter, the electrodes (Electrode for FES, Nihonseisen) were implanted onto the fascia of the left TA muscle, tunneled subcutaneously and exteriorized at the level of the scapulae,¹⁷ and connected to a pulse generator (PulseCure, OG Giken). Electrical stimulation was started on the day after electrode implantation and continued for 5 days with a 0.3-ms stimulus width, 50-Hz stimulus frequency, and 0.1-V stimulus strength, which was far below the threshold of TA muscle contraction. The threshold of TA muscle contraction in this experimental condition detected by electromyogram was 1.1 ± 0.2 V (data not shown). One day after the period of electrical stimulation, animals were anesthetized, blood flow was measured, and then the

animals were killed for the procurement of bilateral TA muscles for further evaluations.

Animals were divided into 3 groups. In the first group (5 animals), TA muscles were continuously stimulated for 5 days after the dissection and excision of the left FA. In the second group (4 animals), electrodes were implanted after the dissection and excision of the left FA, but TA muscles were not electrically stimulated. The third group (6 animals) received a sham operation of the left FA and no electrical stimulation despite the implantation of electrodes. The contralateral hindlimb of each animal served as the control.

Immunohistochemical Analysis

For detection of VEGF protein, muscle specimens were fixed with 10% formaldehyde, preincubated with 1% BSA for 30 minutes, and then incubated with rabbit polyclonal anti-VEGF antibody (1 μ g/mL) (Santa Cruz Biotechnology) for 30 minutes at room temperature. Thereafter, the specimens were stained by the ABC method (Elite, Vector Laboratories, Inc).

Determination of Capillary Density

Numbers of capillaries and muscle fibers were counted in at least 8 different fields, and capillary density was obtained by the calculation of capillary number/fiber area.

Calculations and Statistical Analysis

The statistical significance of differences in the results was evaluated by use of unpaired ANOVA, and a value of $P < 0.05$ was accepted as statistically significant.

Results

Induction of VEGF mRNA by Electrical Stimulation

To determine whether electrical stimulation affects the expression of VEGF mRNA, we exposed cultured skeletal muscle cells to electrical pulse stimulation for 24 hours at various frequencies and 1.0 V strength, which did not cause their contraction or interfere with their spontaneous contraction. Northern blot analysis revealed that VEGF mRNA was augmented at specific frequencies. In murine skeletal muscle cell line C2C12 cells, the expression of VEGF mRNA was significantly augmented at the narrow range of frequencies around 50 Hz (Figure 1A). This frequency of electrical stimulation augmented the expression of VEGF mRNA in primary culture of rat skeletal muscle cells as well (data not shown). Although 50-Hz electrical stimulation seemed to be optimal for the expression of VEGF mRNA in skeletal muscle cells, it did not augment the expression of VEGF mRNA in rat aortic smooth muscle cells (Figure 1B) or human fibroblasts (data not shown). Instead, the expression of VEGF mRNA was augmented at 24-Hz stimulation in rat aortic smooth muscle cells (Figure 1B) as well as human pulmonary artery smooth muscle cells (data not shown). A time-course experiment revealed that VEGF mRNA was increased 4.8-fold in skeletal muscle cells and 4.5-fold in smooth muscle cells at the 8-hour time point at the proper frequency. To exclude the possibility that substances that might be released from the electrodes into the medium affected the expression of VEGF mRNA, we electrically agitated the medium and exposed it to the cells. We did not see any augmentation of the expression of VEGF mRNA by this procedure (data not shown). The expression of VEGF receptor-2 (KDR/Flk-1) mRNA in BCECs was not affected by electrical stimulation at various frequencies (Figure 1C).

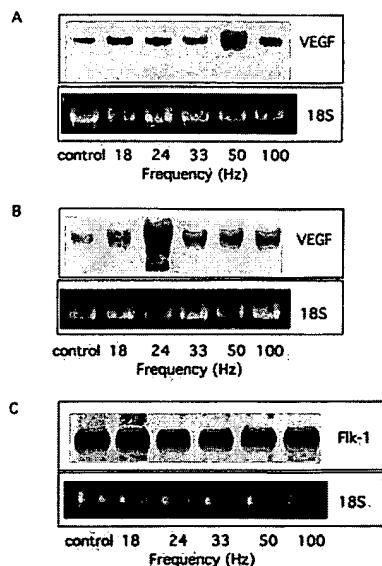


Figure 1. Expression of VEGF mRNA in cultured smooth muscle cells and skeletal muscle cells and flk-1 mRNA in BCECs. Cells were electrically stimulated for 24 hours at indicated frequencies. Thereafter, total RNA was extracted, and Northern blotting for VEGF mRNA or flk-1 mRNA was performed. A, murine skeletal muscle cell line, C2C12 cells; B, rat aortic smooth muscle cells; C, BCECs. Results are representative of at least 3 independent experiments.

The level of expression of VEGF mRNA was determined by the transcription rate of VEGF gene and/or the stability of VEGF mRNA. Therefore, we used actinomycin-D (Act-D) to evaluate the stability of VEGF mRNA. As shown in Figure 2, the half-lives of VEGF mRNA, which were calculated by drawing the best-fit linear curve on a log-linear plot of the percentage of RNA remaining versus time in Act-D-treated cells, were 0.99 hour for electrically stimulated and 1.12 hour

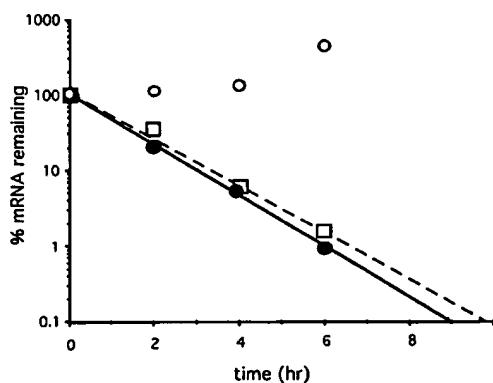


Figure 2. Analysis for stability of VEGF mRNA in C2C12 cells. Stability of VEGF mRNA was analyzed by addition of Act-D (4 μ mol/L). Total RNA was extracted at various time points, and Northern blotting for VEGF mRNA was performed. Quantification of VEGF mRNA was performed with FLA 2000 Image Analyzer, and amount of VEGF mRNA was corrected for loading differences by amount of ribosomal RNA. Plotted data are expressed as % RNA remaining, which is representative of 3 independent experiments. O, Electrical stimulation alone; □, Act-D without electrical stimulation; ●, Act-D with electrical stimulation. Half-life of VEGF mRNA in Act-D-treated cells was calculated by drawing best-fit linear curve on log-linear plot of % RNA remaining vs time.

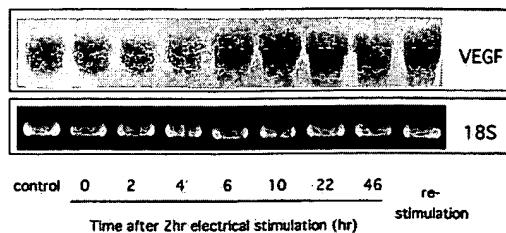


Figure 3. Time course of induction of VEGF mRNA by transient electrical stimulation. C2C12 cells were exposed to 2 hours of electrical stimulation, and total RNA was extracted at indicated time points after transient electrical stimulation. Control indicates before transient stimulation; restimulation, cells were stimulated a second time for 2 hours at 22 hours after first stimulation. Total RNA was extracted at 22 hours after second stimulation. Results are representative of at least 3 independent experiments.

without electrical stimulation. Thus, the electrical stimulation did not affect the stability of VEGF mRNA, indicating that the augmentation of VEGF mRNA by electrical stimulation was at the transcriptional level.

Next, we examined the effect of transient electrical stimulation. Cells were exposed to 2 hours of electrical stimulation, and then the total RNA was harvested at 22 hours after the transient stimulation. The results showed that the induction of VEGF mRNA was observed 22 hours after the transient stimulation, and the level of its expression was almost identical to that of the continuous electrical stimulation for 24 hours (Figure 3). Because VEGF mRNA returned to the basal level by 46 hours after a 2-hour electrical stimulation, the second stimulation could increase VEGF mRNA to a level comparable to the first stimulation. Thus, the transient electrical stimulation was equally effective and was repeatable.

Synthesis and Secretion of VEGF Protein

The synthesis and secretion of VEGF protein into the medium was analyzed by ELISA. VEGF in the medium of both C2C12 cells and rat aortic smooth muscle cells was increased by electrical stimulation (Figure 4A). These stimulatory effects were observed to increase VEGF mRNA at the same specific frequencies in these cells. The elevation of VEGF protein in the medium was observed as early as 12 hours and reached its peak at 48 hours (Figure 4B). The conditioned medium of electrical stimulation augmented the growth of BCECs as much as the medium supplemented with 10 ng/mL of human recombinant VEGF (Figure 4C). Although proteins other than VEGF might also be responsible for the growth of BCECs, the concentration of VEGF in conditioned medium determined by ELISA suggested that most of the effects on the growth of BCECs were derived from VEGF.

Rat Model of Ischemic Hindlimb

We used the ischemic hindlimb animal model to investigate whether electrical stimulation might induce angiogenesis and increase blood flow in an ischemic limb. Blood flow was measured in TA muscles of ischemic and contralateral limbs. The percentage of blood flow in experimental muscles versus contralateral muscles is shown in Figure 5. Blood flow in the ischemic limb on day 7 after the left FA excision was

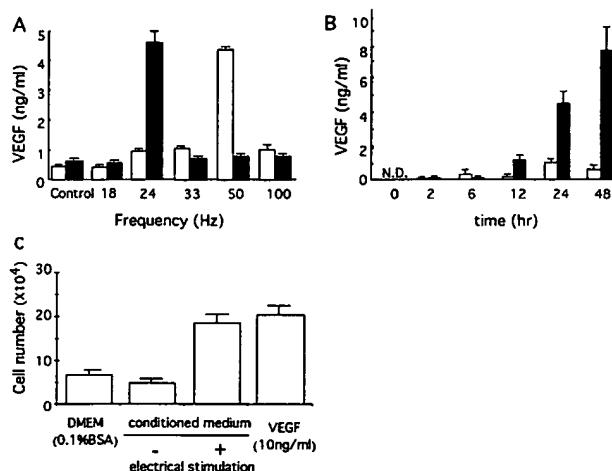


Figure 4. Synthesis, secretion, and biological activity of VEGF protein. Medium was collected during continuous electrical stimulation, and secreted VEGF protein in medium was determined with ELISA. A, Rat aortic smooth muscle cells (solid columns) and C2C12 cells (open columns) were exposed to indicated frequencies for 24 hours. B, C2C12 cells were stimulated electrically, and conditioned medium was collected at indicated time points (solid columns). C2C12 cells without electrical stimulation (open columns). C, Conditioned medium of C2C12 cells with or without electrical stimulation was collected. BCECs were plated in 60-mm plastic dishes at a density of 5×10^4 cells/dish and incubated in DMEM containing 10% FCS for 6 hours. Then culture medium was replaced as indicated. Cell number was determined after 48-hour incubation. Values are mean \pm SD of triplicate experiments.

significantly lower than that in the contralateral limb as well as in the sham-operated animals. Whereas blood flow in the ischemic limb on day 14 after the left FA excision did not

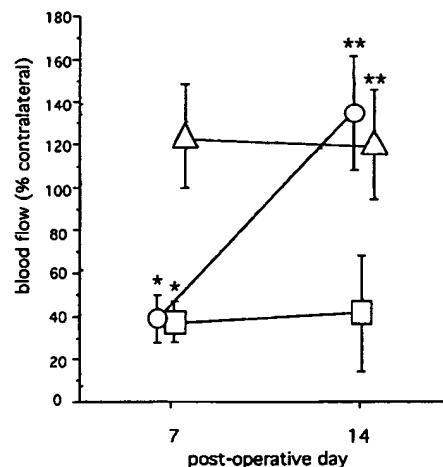


Figure 5. Blood flow in rat TA muscles was analyzed at postoperative days 7 and 14. Electrical stimulation was continued from postoperative day 8 to day 12. Values are expressed as a percentage of blood flow in experimental muscles versus contralateral muscles. At postoperative day 7, blood flow in FA excision alone (□) and FA excision with electrical stimulation (○) were significantly lower than that in sham-operated limbs (△) (* $P<0.01$). At postoperative day 14, values in electrically stimulated and sham-operated limbs were significantly higher than those in FA excision alone (** $P<0.01$), and there was no significant difference in blood flow between stimulated and sham-operated limbs. All values are given as mean \pm SD.

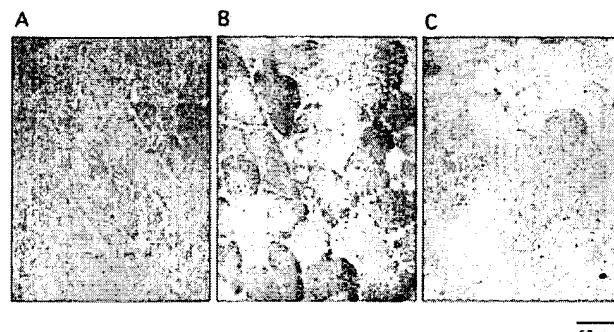


Figure 6. Immunohistochemical stains for VEGF protein in rat TA muscles. For detection of VEGF protein, muscle specimens were fixed with 10% formaldehyde, preincubated with 1% BSA for 30 minutes, and then incubated with rabbit polyclonal anti-VEGF antibody (1 μ g/mL) for 30 minutes at room temperature. Thereafter, specimens were stained by ABC method. A, Contralateral TA muscle; B, TA muscles after 5 days of electrical stimulation; C, unstimulated TA muscle with FA excised.

increase without any stimulation, continuous electrical stimulation significantly increased blood flow in the TA muscle of the ischemic limb. In addition, rats tolerated this procedure very well, and there was no muscle contraction, muscle injury, or restriction in movement.

Immunostaining of TA muscles with polyclonal anti-VEGF antibody revealed a significant increase of VEGF protein in the muscle fibers of electrically stimulated TA muscles compared with unstimulated muscles or contralateral

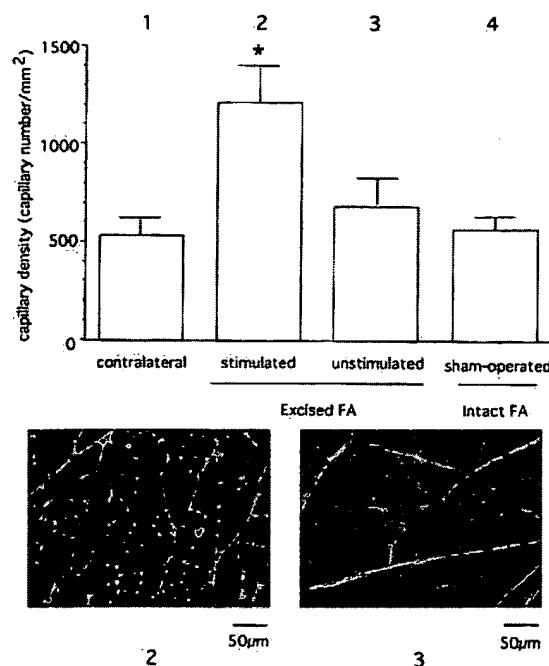


Figure 7. Capillary density in rat TA muscles. Number of capillaries was counted in at least 8 different fields, and capillary density was obtained by calculation of capillary/muscle fiber area. Whereas difference between contralateral TA muscles (lane 1) and unstimulated (lane 3) or sham-operated (lane 4) muscles was not significant, that between contralateral (lane 1) and stimulated (lane 2) muscles was significant (* $P<0.01$). Representative images of lane 2 (FA excised and electrically stimulated) and lane 3 are shown.

TA muscles (Figure 6). This increase of VEGF protein was found only in the area between the electrodes on TA muscle. Capillary density of the stimulated muscles was increased ≈ 2.5 -fold compared with the contralateral muscles, whereas unstimulated animals as well as sham-operated animals had no significant difference between ischemic and contralateral muscles (Figure 7).

Discussion

Our present study demonstrated that direct electrical stimulation of cultured skeletal muscle cells induced the expression of VEGF. Moreover, the low-voltage electrical stimulation of ischemic skeletal muscle augmented de novo synthesis of VEGF protein in the muscle, induced regional angiogenesis, and restored blood flow in ischemic muscle. Electrical stimulation at 100 Hz did not increase the capillary density in animals (data not shown). This also implied that metabolic activation could not simply explain the augmentation of VEGF synthesis in the muscle. It was interesting that the optimal electrical frequency seemed to vary between individual cell types, ie, 50 Hz for skeletal muscle cells and 24 Hz for smooth muscle cells. Frequency-dependent gene expression or protein synthesis in insulin-like growth factor-2 of osteosarcoma cells¹⁸ and constitutive NO synthase of cardiomyocytes¹⁹ have been described previously. Although the mechanism of the frequency dependence of VEGF induction is obscure at the moment, this characteristic will allow us to restrict the induction of VEGF to skeletal muscle. Two reports describe the augmentation of VEGF synthesis after in vivo electrical stimulation: in one, skeletal muscle was stimulated at 10 Hz and 3 to 4 V, which induced muscle contraction¹⁷; in the other, a motor nerve was stimulated at 6 to 10 Hz, which also induced muscle contraction.²⁰ Because the frequencies used were far below the optimal value for VEGF induction in skeletal muscle, the effects of these studies on the synthesis of VEGF might not be derived from muscle contraction.

Induction of angiogenesis in skeletal muscle by electrical stimulation has been described.²¹⁻²⁵ However, the aim of electrical stimulation in those reports was to produce maximal muscle contraction. Indeed, they used an electrical strength of >2.0 V for the stimulation. It was hypothesized that hypoxia caused by muscle contraction was relevant to the increase of capillary density of skeletal muscles in their model.²⁵ Nevertheless, strenuous electrical stimulation might be harmful, fail to restore blood flow, increase muscle atrophy, and worsen fatigue.²⁶ Here, we used 0.1 V of electrical strength for stimulation of rat TA muscle. This strength of electrical stimulation was 10% of the threshold of muscle contraction and was well tolerated. We therefore propose that this procedure can be applicable as a simple and practical method of therapeutic angiogenesis.

Transcutaneous electrical nerve stimulation (TENS) has been used in the clinical field for pain control. Interestingly, TENS is highly effective in acute musculoskeletal pain as well as pain from peripheral vascular disease and from angina pectoris. However, the reasons for pain relief from TENS are largely unknown at present.²⁷ Because TENS is primarily nerve stimulation and a high frequency in the range of 60 to

100 Hz is generally considered conventional for this treatment, it is not known whether conventional TENS induces regional angiogenesis.

Our results indicated that electrical stimulation augmented VEGF mRNA via transactivation of the VEGF gene. However, the mechanism of how low-voltage electrical stimulation induces the expression of VEGF gene is unknown. VEGF can be induced by various stimuli. Among them, hypoxia is thought to be one of the most important stimuli for the expression of the VEGF gene. Transactivation of the VEGF gene in hypoxia is mediated by hypoxia-inducible factor-1, which binds to the hypoxia responsible element in the 5' flanking region of the VEGF gene. Heme-containing protein works as a sensor of oxygen, and hypoxia, as well as nickel or cobalt, induces hypoxia-inducible factor-1 by activating this sensing system.²⁸ We used nickel to mimic the hypoxic condition and found that the electrical stimulation reinforced the expression of VEGF in the hypoxic condition (data not shown). This notion is consistent with the in vivo experimental results showing that electrical stimulation effectively augmented VEGF synthesis in the ischemic skeletal muscle.

Gene therapy using VEGF is thought to be relevant for therapeutic angiogenesis, and a clinical trial is now progressing in the United States.⁷ However, gene therapy is not always available. The important point on this issue is that the expression of VEGF is not restricted to a specific cell type; rather, it is synthesized by a variety of cell types surrounding ECs. If one could force cells in the ischemic area to synthesize a sufficient amount of endogenous VEGF protein, synthesized VEGF would promote local angiogenesis and salvage the ischemic area. Our results reinforce the above idea. We propose that it would be valuable to consider this procedure for clinical application.

References

- Pepper MS. Manipulating angiogenesis from basic science to the bedside. *Arterioscler Thromb Vasc Biol.* 1997;17:605-619.
- Baffour R, Berman J, Garb JL, Rhee SW, Kaufman J, Friedmann P. Enhanced angiogenesis and growth of collaterals by in vivo administration of recombinant basic fibroblast growth factor in a rabbit model of acute lower limb ischemia: dose-dependent effect of basic fibroblast growth factor. *J Vasc Surg.* 1992;16:181-191.
- Takeshita S, Zheng LP, Brogi E, Kearney M, Pu LQ, Bunting S, Ferrara N, Symes JF, Isner JM. Therapeutic angiogenesis: a single intra-arterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hindlimb model. *J Clin Invest.* 1994;93:662-670.
- Hariwala M, Horowitz JR, Esakof D, Sheriff DD, Walter DH, Keyt B, Isner JM, Symes JF. VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts. *J Surg Res.* 1996;63:77-82.
- Banai S, Jaklitsch MT, Shou M, Lazarus DF, Schinowitz M, Biro S, Epstein SE, Unger EF. Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. *Circulation.* 1994;89:2183-2189.
- Yanagisawa-Miwa A, Uchida Y, Nakamura F, Tomaru T, Kido H, Kamijo T, Sugimoto T, Kaji K, Utsuyama M, Kurashima C, Ito H. Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. *Science.* 1992;257:1401-1403.
- Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K, Isner JM. Constitutive expression of phVEGF₁₆₅ after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation.* 1998;97:1114-1123.

8. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*. 1989;246:1306–1309.
9. Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature*. 1992;359:845–848.
10. Ferrara N, Houck K, Jakeman L, Leung DW. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocrinol Rev*. 1992;13:18–32.
11. Sato Y, Shimada T, Takaki R. Autocrinological role of basic fibroblast growth factor on tube formation of vascular endothelial cells in vitro. *Biochem Biophys Res Commun*. 1991;180:1098–1102.
12. Brevet A, Pinto E. Myosin synthesis increased by electrical stimulation of skeletal muscle cell cultures. *Science*. 1976;193:1152–1154.
13. Iwasaka C, Tanaka K, Abe M, Sato Y. Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metalloproteinase-1 and the migration of vascular endothelial cells. *J Cell Physiol*. 1996;169:522–531.
14. Takemoto S, Isshiki T, Sato T. Increased expression of direct gene transfer into skeletal muscle observed after acute ischemic injury in rats. *Lab Invest*. 1996;74:1061–1065.
15. Aukland K, Bower BF, Berliner RW. Measurement of local blood flow with hydrogen gas. *Circ Res*. 1964;14:164–187.
16. Hori K, Zhang QH, Li HC, Saito S, Sato Y. Timing of cancer chemotherapy based on circadian variations in tumor tissue blood flow. *Int J Cancer*. 1996;65:360–364.
17. Hang J, Kong L, Gu L, Adir TH. VEGF gene expression is upregulated in electrically stimulated rat skeletal muscle. *Am J Physiol*. 1995;269:H1827–H1831.
18. Fitzsimmons RJ, Strong DD, Mohan S, Baylink D. Low-amplitude, low-frequency electric field-stimulated bone cell proliferation may in part be mediated by increased IGF-II release. *J Cell Physiol*. 1992;150:84–89.
19. Kaye DM, Wiviott SD, Balligand JL, Simmons WW, Smith TW, Kelly RA. Frequency-dependent activation of a constitutive nitric oxide synthase and regulation of contractile function in adult rat ventricular myocytes. *Circ Res*. 1996;78:217–224.
20. Annex BH, Torgan CE, Lin P, Taylor DA, Thompson MA, Peters KG, Kraus WE. Introduction and maintenance of increased VEGF protein by chronic motor nerve stimulation in skeletal muscle. *Am J Physiol*. 1998;274:H860–H867.
21. Brown MD, Cotter MA, Hudlicka O, Vrbova G. The effect of different patterns of muscle activity on capillary density, mechanical properties and structure of slow and fast rabbit muscles. *Pflugers Arch*. 1976;361:241–250.
22. Hudlicka O, Tyler KR. The effect of long-term intermittent high-frequency stimulation on capillary density and fibre profiles in rabbit fast muscles. *J Physiol*. 1984;353:435–445.
23. Hudlicka O, Fronk K. Effect of long-term electrical stimulation of rabbit fast muscles on the reactivity of their supplying arteries. *J Vasc Res*. 1992;29:13–19.
24. Mathieu-Costello O, Agey PJ, Wu L, Hang J, Adair TH. Capillary-to-fiber surface ratio in rat fast-twitch hindlimb muscle after chronic electrical stimulation. *J Appl Physiol*. 1996;80:904–909.
25. Hudlicka O, Price S. The role of blood flow and/or muscle hypoxia in capillary growth in chronically stimulated fast muscles. *Pflugers Arch*. 1990;417:67–72.
26. Hudlicka O, Brown MD, Egginton S, Dawson JM. Effect of long-term electrical stimulation on vascular supply and fatigue in chronically ischemic muscles. *J Appl Physiol*. 1994;77:1317–1324.
27. Long DM. Fifteen years of transcutaneous electrical stimulation for pain control. *Stereotact Funct Neurosurg*. 1991;56:2–19.
28. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol*. 1996;16:4604–4613.



I hereby Certify that this Correspondence is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to:
Commissioner for Patents, P.O. Box 1450, Alexandria,
Virginia 22313-1450, on January 4, 2005

Name: Shinichi Kanno
Signature: Shinichi Kanno
Date of Signature: January 4, 2005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No. : 09/858,036
Applicant(s) : Kanno, et al.
Filed : May 15, 2001
Title : Method For Inducing Angiogenesis By Electrical Stimulation Of Muscles
Docket No. : 31090.0015

37 C.F.R. 1.132 Declaration

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The undersigned, Shinichi Kanno, declares:

1. That he is Assistant professor of Surgery at the University of Pittsburgh School of Medicine.
2. That he is the inventor on the above-referenced patent application.
3. That he is familiar with the Office Action mailed on October 4, 2004 in which claims 1-15 were rejected as anticipated by Donovan et al. (Patent Application Publication No. 2002/0010492), which claims priority to Provisional Application No. 60/187,280, filed March 6, 2000.

Rule 1.132 Declaration by Dr. S. Kanno

Page 2

4. That he has separately presented a Rule 1.131 declaration that the present invention was made at least as early as May 25, 1999, as evidenced by the publication Kanno, et al. (*Circulation*. (May 25, 1999) 99:2682-2687).

5. That this publication is the inventors' own work and that all authors listed who are not named inventors on the instant application did not make inventive contribution to the present application.

6. That all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued therefrom.

Respectfully submitted,

12/20/00

Date



Dr. Shinichi Kanno

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.